IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
William P. Spencer et al.	Group Art Unit: 1621
Application No.: 10/805,386	Examiner: Deborah D. Carr
Filed: March 22, 2004	Confirmation No.: 1731
For: ESTERIFIED FATTY ACID COMPOSITION	

DECLARATION BY ROBERT HESSLINK UNDER 37 C.F.R. § 1.132

Commissioner for Patents
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Sir:

I, Dr. Robert Hesslink, hereby state as follows:

1. I reside at 12305 Brianwood Drive, Riverside, CA 92503.

2. I am a citizen of the United States of America.

3. My educational background is as follows:
B.A. Physical Education, California State University, Northridge
M.S.T. Physical Education, Portland State University, OR
Sc.D. Applied Anatomy & Physiology, Boston University, MA.

4. I am currently employed at Imagenetix, as Director, Research & Development.

5. My research interests are in the field of Nutrition, Lipid Metabolism, Exercise Physiology, Environmental Physiology.

6. A copy of my complete Curriculum Vitae is attached as Exhibit A.

7. I am not an inventor of U.S. Patent Application Serial No. 10/805,386. However, I am familiar with the patent application, and the experimental data that

has been generated with regard to the mechanism of action of the esterified fatty acids of the application, as well as their use to treat certain disease conditions.

Background/ Mechanism of Action of Esterified Fatty Acids

8. Arachidonic acid is a constituent of the SN-2 domain of membrane phospholipids (FitzGerald, Garrett, The cardiovascular choreography of COXs. Japanese Circulation Society, 67th Scientific Session, March 28-30, 2003, Fukuoka, Japan) and is released by the action of phospholipases that are mobilized within the membrane by elevations in intracellular calcium. The pathogenesis of numerous diseases and conditions, including cardiovascular, pulmonary, inflammatory and thromboembolic diseases, can be related to arachidonic acid (AA) metabolites. In particular, thromboxane A2 (TXA2) is produced by the action of thromboxane synthase on prostaglandin endoperoxide 112 (PGH2). Esterified fatty acids are composed of a fatty alcohol (hexadecanol) and a fatty acid, which are linked together via an esterification process. The present esterified fatty acids have been shown *in vitro* to inhibit thromboxane A2 production. The reduction in thromboxane A2 production was confirmed in a separate experiment in which thromboxane synthase receptor inhibition was evident after exposure to the esterified fatty acids *in vitro*. This data demonstrates that the esterified fatty acids play a vital role in the regulation of thromboxane A2 production from arachidonic acid metabolism at the cell membrane. In addition, esterified fatty acids were shown to inhibit PGE2 production via COX-2 enyzme inhibition. In a classic tracer study, the present esterified fatty acids were shown to be localized in the cell membrane of the liver and white blood cells (Gallaher DD., Incorporation of Myristyl Hexadecanol and its Metabolites into Membranes of Tissues and White Blood Cells of Rats, Final Report, January 3, 2006; discussed in greater detail below).

9. Local and systemic inflammation can be modulated by a variety of pro-inflammatory pathogens. Often these pathogens are a result of bacteria found in diseases of the mouth. *Actinomyces viscosus* has been reported as associated with gingivitis (Hiratsuka M, Shibata Y. Effect of actinomyces viscosus on the product of prostaglandin E2 and thromboxane B2 in macrophages. Nichidai Koko Kagaku,

1989, 15(2): 79-85; Tynelius-Bratthall G, Ellen RP. Fluctuations in crevicular and salivary anti-A. viscosus antibody levels in response to treatment of gingivitis. J Clin Periodontol 1985, 12(9): 762-73). This bacteria is thought to stimulate the release of arachidonic acid from cellular membranes and the eventual secretion of thromboxane A2 and PGE2. The continued production of these pro-inflammatory pathogens can lead to significant tissue damage leading to periodontal disease.

Study to determine the metabolic fate of esterified fatty acids

10. A study was performed to determine the metabolic fate of esterified fatty acids, once ingested. A radioactively labeled esterified fatty acid, myristyl hexadecanol (MHD), was synthesized using radioactive ^{14}C-hexadecanol and ^{3}H-myristic acid. The radiolabeled MHD was administered to rats orally for seven days, and then tissues (brain, liver, kidney, muscle, and fat) and blood were collected. The membrane material (microsomes) and intracellular material (cytosol) were collected, and lipids (fats) were extracted. The materials were separated into different fractions, and the radioactivity in each fraction measured. In addition, white blood cells from blood the microsomes were isolated and analyzed.

11. Five rats were given orally a radioactively labeled MHD twice a day for seven days. The radiolabeled MHD was synthesized from ^{3}H-myristic acid and ^{14}C-hexadecanol (cetyl alcohol), which allowed us to follow the metabolic fate of the two lipid components of MHD separately. The rats were then killed, and the liver, kidney, adipose, muscle, brain tissues and blood were harvested. In addition, mononuclear cells (lymphocytes and other mononuclear cells) were isolated from whole blood by centrifugation, using a polysucrose solution of density 1.083 g/mL.

12. The tissues were homogenized and centrifuged to obtain a membrane fraction (microsomes) and the aqueous part of the cells (cytosol). The different lipid fractions in the membranes and cytosol were separated by thin layer chromatography, and the radioactivity in these different fractions determined to indicate the form of the MHD in the membranes or cytosol, *e.g.* as fatty acids (as part of phospholipids and cholesterol esters), MHD, hexadecanol, or other lipids.

13. In summary, the following was discovered:

- Radioactivity was found in both the microsomes and the cytosol, indicating that MHD and its metabolites disperse widely within the cells.
- Most of the radioactivity was found in the phospholipids and the triacylglycerols. This was true in both the microsomes and the cytosol.
- The sites with the greatest proportion of intact MHD were the liver and white blood cells, which was present in both microsomes and cytosol.
- No measurable radioactivity was found in the brain.

14. These results indicate that metabolites of the MHD are the major lipids present in the microsomes and the cytosol of tissues and white blood cells. In both the microsomes and the cytosol, only small proportions (<5% of total radiolabel) or no intact MHD was present. The majority of the ^{14}C and ^{3}H radiolabels were present in phospholipids and triacylglycerol in both the microsomes and cytosol. This finding suggests that the MHD was hydrolyzed to hexadecanol and myristic acid, and that the hexadecanol was subsequently oxidized to palmitic acid. Both myristic and palmitic acids were then incorporated into phospholipids and triacylglycerols. An enzymatic activity capable of oxidizing hexadecanol, referred to as fatty alcohol:NAD^{+} oxidoreductase, has been identified in rat liver. This likelihood is reinforced by the finding of ^{14}C radiolabel in the hexadecanol + fatty acid fraction, as ^{14}C label in this fraction almost certainly would arise only from the hydrolysis of MHD.

15. Additionally, some of the fatty acids were esterified to cholesterol. A small proportion of radiolabel from both ^{14}C and ^{3}H labels was detected in the cholesterol fraction. This strongly suggests that a portion of the myristic acid and hexadecanol (or, more likely, its oxidation product palmitic acid), are being oxidized by β-oxidation and a quantity of the acetyl CoA thus generated is being used to synthesize cholesterol. MHD appears to be mostly metabolized to fatty acids, which are then incorporated into fatty acid-containing lipids, such as phospholipids, triacylglycerol, and cholesterol esters. Further, these radiolabeled fatty acid-

containing lipids are present in both the microsomes and the cytosol, indicating a wide cellular distribution of the metabolites.

16. A high proportion of intact MHD present in the white blood cell microsomes was also noted, >4% for both the 14C and ^{3}H labels. Given the significant role white blood cells play in the inflammation response, the presence of MHD in the membrane may alter the secretion of cytokines or another aspect of their biology in a way that would explain the noted biological effect of esterified fatty acids on thromboxane A2 production, thromboxane synthase receptor inhibition and COX-2 inhibition.

Study to evaluate Anti-Infective Activity

17. The compounds of the present invention were evaluated for anti-infective activity against *Actinomyces viscosus* and *Staphylococcus aureus*, Methicillin resistant, at 8 concentrations starting at 100 µg/ml and decreasing by 3 fold dilution to a final concentration of 0.03 µg/ml. Esterified fatty acids inhibited the growth of *Actinomyces viscosus* at concentrations > 10 µg/ml, and *Staphylococcus aureus* at the 100 µ/m1 concentration.

Effect of Esterified Fatty Acids on Periodontal Disease

18. Periodontitis is a local inflammation that occurs because of host response against specific microorganisms and eventually leads to the tissue destruction and systemic complications. The etiology of this infectious disease is specific Gram-negative microorganisms, such as *Porphyromonas gingivalis* and *Tannerella forsythensis*. While the etiology of periodontitis is bacterial, it is clear that the pathogenesis is mediated by the host response. Once periodontal inflammation is initiated, the cascade of inflammatory events includes an amplified loop until the infection is contained and injury is confined. The early actions of the host response are later replaced by mechanisms that are more specific and eventually become redundant. Thus, it is important to limit the response and to allow the inflammation to resolve. While it has been shown that many molecules ("on signals") participate in the initiation and development of the host defense mechanisms, recent paradigm in

periodontal disease pathogenesis emphasizes the importance of counterregulatory molecules ("off signals") in the resolution of inflammatory response to control its magnitude and duration.

19. Several inflammatory mediators such as cytokines, chemokines, and metalloproteases are associated with periodontal disease, and prominent among these are the arachidonic acid-derived products, including leukotriene and prostaglandin (PG)E_2. Many of the pathophysiological events that occur in periodontal diseases can be explained largely by the activities of lipid mediators. For example, LTB_4, a well-appreciated and potent chemoattractant, also initiates the accumulation of leukocytes within inflamed sites, stimulates the release of granule-associated enzymes, and was recently found to stimulate bone resorption. PGE_2 is a very potent stimulator of bone loss, which is held to be a hallmark of periodontal disease. PGE_2 is also noted for its ability to directly mediate vasodilatation, increase vascular permeability, enhance pain perception by bradykinin, and histamine, alter connective tissue metabolism, and enhance osteoclastic bone resorption. The levels of PGE_2 are significantly elevated in the crevicular fluid of patients with periodontal infections.

20. Positive results in the treatment of periodontal disease were obtained with the present esterified fatty acids using a rabbit model. These results are consistent with the *in vitro* finding that the esterified fatty acids inhibited growth of *Actinomyces viscosus* (ATCC 15987) at an effective concentration of 10 ug/ml. (This experiment was performed *in vitro* using a standardized test medium of heart brain broth (MDS Pharma Services, Report #1014123, June 30, 2004, Study Directors: Cheng F-C, Lin C-C, Study). The esterified fatty acids inhibit growth of *Actinomyces viscosus*, an important oral microbes common in oral disease. The esterified fatty acids inhibit the production of thromboxane A2, a metabolite of arachidonic acid metabolism vital to inflammation (MDS Pharma Services, Report #1014124, June 30, 2004, Study Directors: Cheng F-C, Lin CC). In addition, esterified fatty acids were shown to inhibit PGE2 production via the COX-2 pathway (MDS Pharma

Services, Report #1074130, March 2006, Study Directors: Chang Kang-Kuang; Lin CC).

21. Rabbits represent a relevant model, in which the physiology and the pathology of periodontal tissues resemble humans with respect to pro-inflammatory and anti-inflammatory mechanisms. It has already been shown that a predictable and reproducible periodontitis can be generated in the rabbits by using silk ligatures accompanied by the topical application of periodontitis-specific microorganism *P. gingivalis.*

22. A study was performed to investigate the topical application of esterified fatty acid complex on ligature-induced periodontitis model in rabbits. Periodontitis was developed in rabbit jaws by placing silk ligatures around the second premolars and topical application of periodontitis-specific pathogen *Porphyromonas gingivalis.* While the first two groups of animals (test) topically received the fatty acid complex formulated medication using two separate doses (1 mg/ml and 10 mg/ml), the last group (control) received a similar placebo agent (olive oil). After the animals were sacrificed at 6 weeks, macroscopic, histological and radiographical evaluations of tissue specimens were made to evaluate the local effects of cetylated fatty acids on the gingival tissues and periodontal disease progression.

23. A total of 15 male New-Zealand White rabbits were equilibrated and housed at the Laboratory Animal Science Center. Animals were randomly assigned into 3 treatment groups as follows:

Group A: Ligature + P. gingivalis + placebo (olive oil);

Group B: Ligature + P. gingivalis + test agent-1 (compound 1) at 1 mg/ml concentration; and

Group C: Ligature + P. gingivalis + test agent-2 (compound 2)10 mg/ml concentration.

24. On the day of experiment, rabbits were anesthetized using xylazine (subcutaneous, 0.25 mL) and ketamine (40 mg/kg, IM) and if necessary intubated and given isoflurane (1-2.5 MAC). Ligatures (3-0 braided silk suture) were placed around the 2nd premolars of both sides of the mandible. Every other day animals were anesthetized using isoflurane to apply the topical medications around the ligatures.

25. *P. gingivalis* (strain A7436) was grown using standard procedures and 10^9 CFU was mixed with carboxy methyl cellulose to form a thick slurry and applied topically to the ligated teeth to induce periodontitis. Following *P. gingivalis* application, test agents (compound 1, compound 2, or placebo) was applied topically to the same areas for six weeks. At these times, the sutures were also checked, and lost or loose sutures were replaced. Olive oil, the carrier for fatty acid complex served as placebo.

26. At the end of the six weeks, animals were euthanized by Pentobarbital overdose (120+mg/kg, iv). After euthanasia, the mandible of each rabbit was dissected free of muscles and soft tissue, keeping the attached gingiva intact with the alveolar bone. The mandible was split into 2 halves from the midline between the central incisors. The left half was taken for morphometric analysis of the bone and the right half was used for histological evaluation of the use of the test and placebo agents in periodontitis. Half of the sectioned mandible was defleshed by immersing in 10% hydrogen peroxide (3-4 days, room temperature). The soft tissue was removed carefully and then the mandible was stained with methylene blue for good visual distinction between the tooth and the bone. Next, the bone level around the second premolar was measured directly by a 0.5 mm calibrated periodontal probe. Measurements were made at three points each, at buccal and lingual sides, for crestal bone level. A mean crestal bone level around the tooth was calculated. Similarly, for the proximal bone level, measurements were made at mesial and distal aspects of the tooth. The measurements were taken from both the buccal and lingual side on both proximal aspects of the second premolar and the mean proximal bone level was calculated by Image Analysis (Image-Pro Plus 4.0, Media

Cybernetics, Silver Spring, MD). The sectioned mandible was mounted and photographed using an inverted microscope at 10X. The captured image was also analyzed as above and the mean crestal bone level around the tooth was calculated in millimeters.

27. The percentage of the tooth within the bone was calculated radiographically using Bjorn technique (Jain *et al.,* 2003). The radiographs were taken with a digital X-ray (Schick Technologies Inc, Long Island City, NY). To quantify bone loss, the length of the tooth from the cusp tip to the apex of the root was measured, as was the length of the tooth structure outside the bone, measured from the cusp tip to the coronal extent of the proximal bone. From this, the percentage of the tooth within the bone was calculated. Bone values are expressed as the percentage of the tooth in the bone (length of tooth in bone x 100 / total length of tooth).

28. For histological analysis, the other half of the mandible was immersed in a volume of Immunocal (Decal Corporation, Tallman, NY, USA) equal to at least 10 times the size of section; solution was replaced every 24 hours for two weeks. After the decalcification, thin sections (5 μm) were cut and sections were either conventionally stained with Hematoxylin-Eosin to identify the cellular composition of the inflammatory infiltrates or with tartrate-resistant acid phosphatase (TRAP) to detect the osteoclastic activity.

29. Figures 1 and 2 show the mandibles of rabbits treated either by ligature and topical *P. gingivalis* + placebo application or ligature and *P. gingivalis* + the esterified fatty acids at two different concentrations. The figure demonstrates the gingival tissue and defleshed bone specimens from buccal and lingual aspects. No obvious and significant differences were found between the three groups, while there was clear evidence of local inflammatory changes in all groups of animals because of ligature and *P. gingivalis* application. Topical delivery of two different doses of esterified fatty acids before *P. gingivalis* application did not show any difference in prevention of both the gingival inflammation and bone destruction compared to

placebo group and no apparent dose-dependent effect was detected between test groups (Groups B and C). Figure 3 shows the quantitative analyses of defleshed bone specimens. The findings demonstrate that all three groups of animals presented a detectable level of bone loss with no significant difference between the placebo and test groups ($p>0.05$).

30. Figure 4 illustrates the radiographic images of the animals in all three groups. Figure 5 shows the percentage of bone loss as calculated by Bjorn technique using these radiographic images. This measurement further confirmed that the cetylated fatty acid application did not show any difference compared to the placebo group with respect to radiographic bone loss.

31. Figure 6 shows the illustration of the technique used for the histomorphometric measurements. Histomorphometric measurements were performed at the ligated site of each tooth. At three points, the linear measurements were made: crestal, mid, and apical third of the alveolar bone. The area measurements were also done on the ligated site of the alveolar bone.

32. In order to quantitatively analyze periodontal disease progression in the rabbit animal model treated with esterified fatty acids as compared to placebo group, the mean value (±standard deviation) of the linear distance and area were calculated for each group (Figure 7). Linear distance was described as the distance from the base of the epithelium to the alveolar crest border at the three chosen levels, the apical, middle, and the coronal third of the root and was expressed as the ratio between the ligated and non-ligated sites. Likewise, area measurements were presented as the proportion of the total area at ligated to the non-ligated aspects of the teeth. Figure 7 shows the comparisons of the linear measurements of the study groups. Although both esterified fatty acids groups showed slightly less bone loss when compared to placebo group there was no significant difference. As shown in Figure 8, there was no difference between the groups when total area of bone calculated.

33. The TRAP stained sections of the ligated and diseased sites of the control group showed disrupted connective tissue and increased inflammatory cell infiltrate especially at the alveolar bone borders. The number of osteoclasts at the apical, middle, and coronal thirds of the root was another variable that was compared between the groups (Figure 9). In the placebo group, there was more osteoclastic activity detected in all sections compared to both test groups. Although both concentrations of esterified fatty acids showed a trend to suspend the osteaoclastic activity compared to placebo, the application of esterified fatty acids at 10mg/ml had a significant ($p<0.05$) preventive effect of osteoclastic activity at coronal and mid third of the root.

34. In view of the above, esterified fatty acids are an effective treatment for cardiovascular conditions, periodontal disease, and psoriasis, as well as other conditions associated with the mechanism of action described herein.

35. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 3-21-2006

Robert Hesslink

Dr. Robert Hesslink

Figure 1

Soft tissue changes

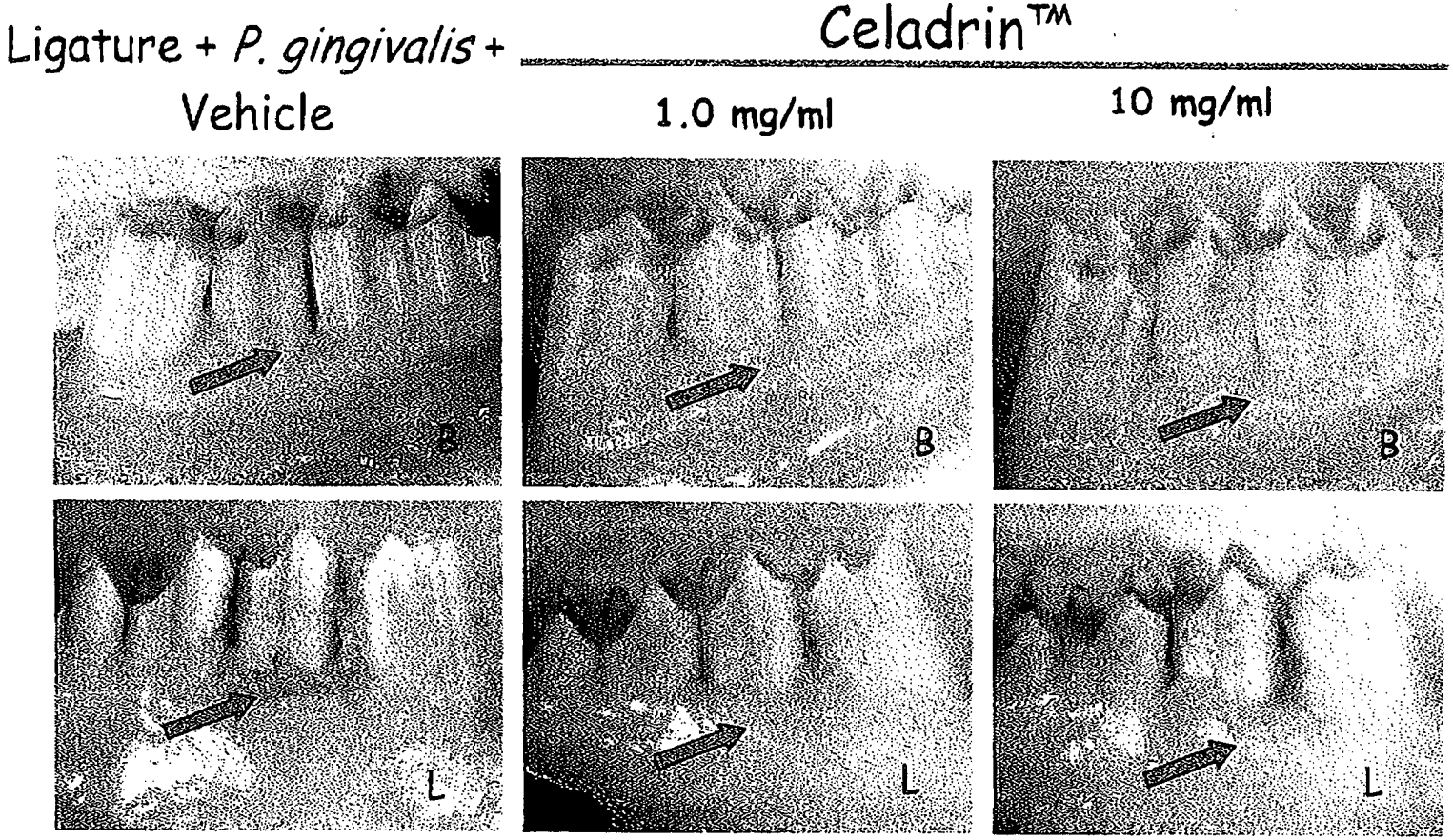


Figure 2

Hard tissue changes

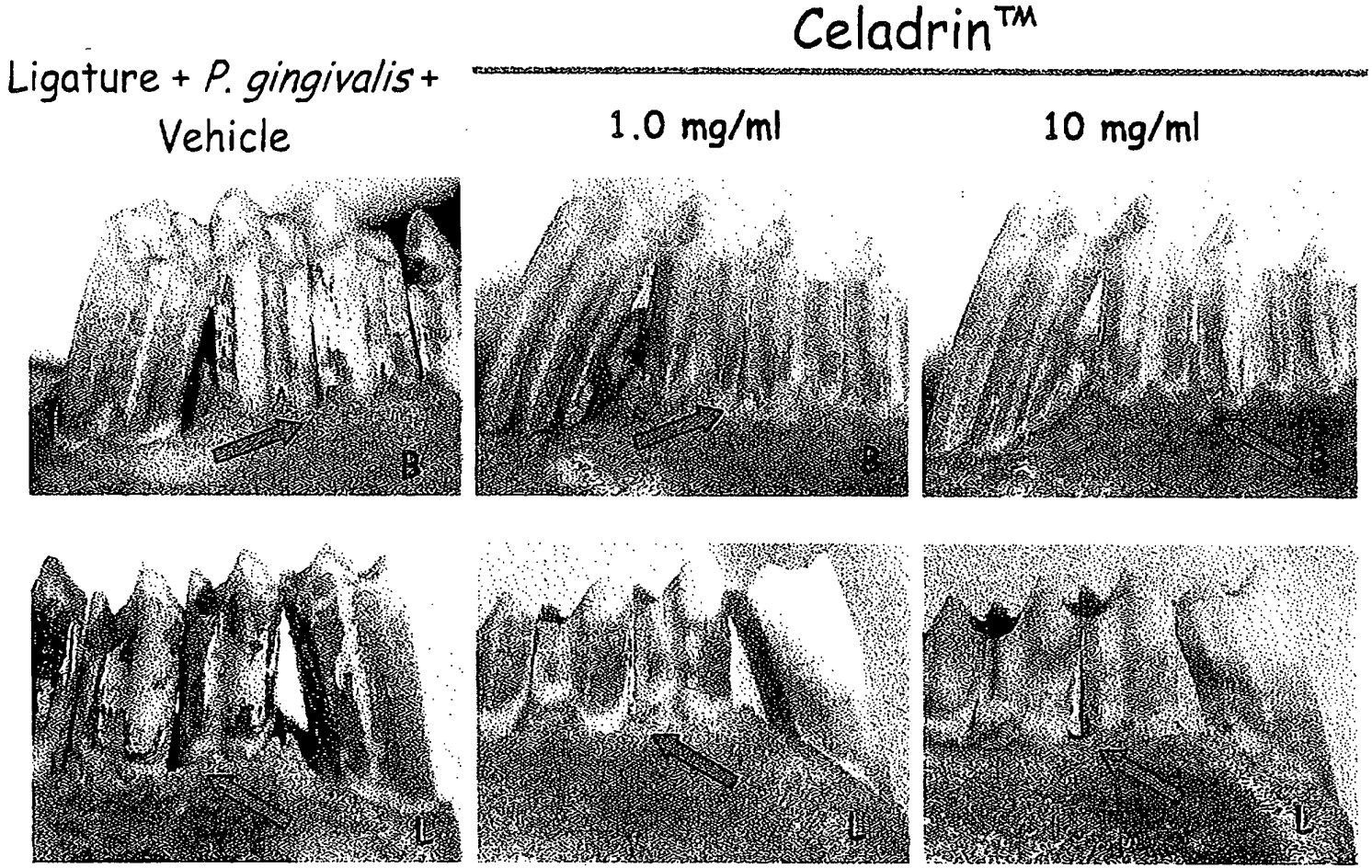


Figure 3

Clinical Bone Loss

(Distance Between Crest and Base of Alveolar Bone)

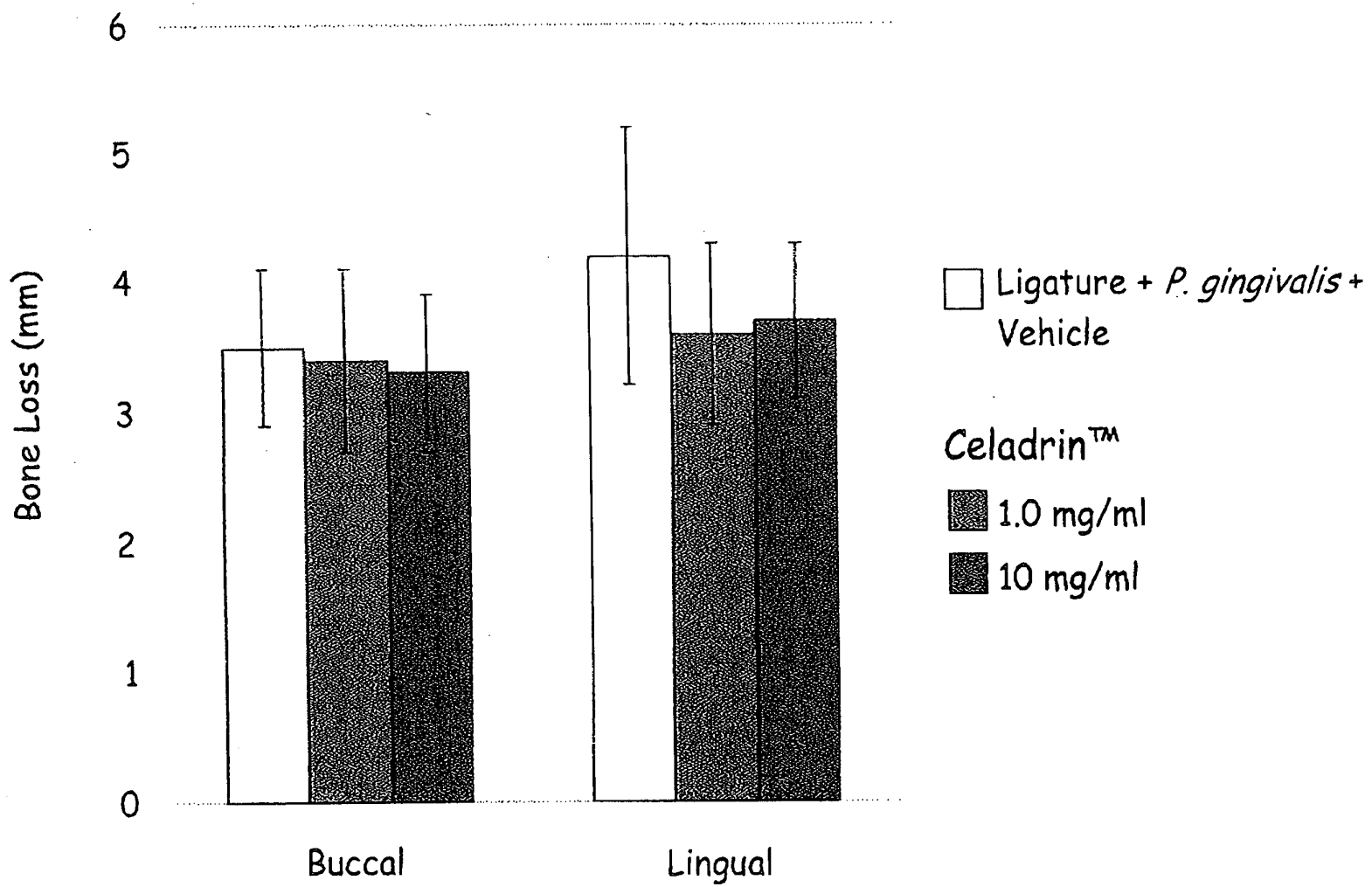


Figure 4

Radiographic Analysis

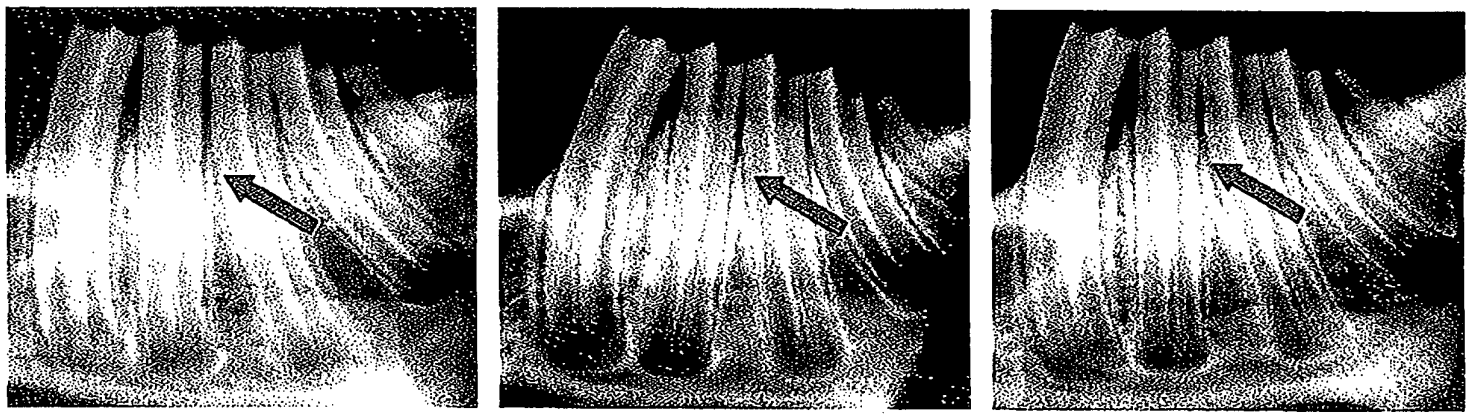


Figure 5

Radiographic Bone Loss

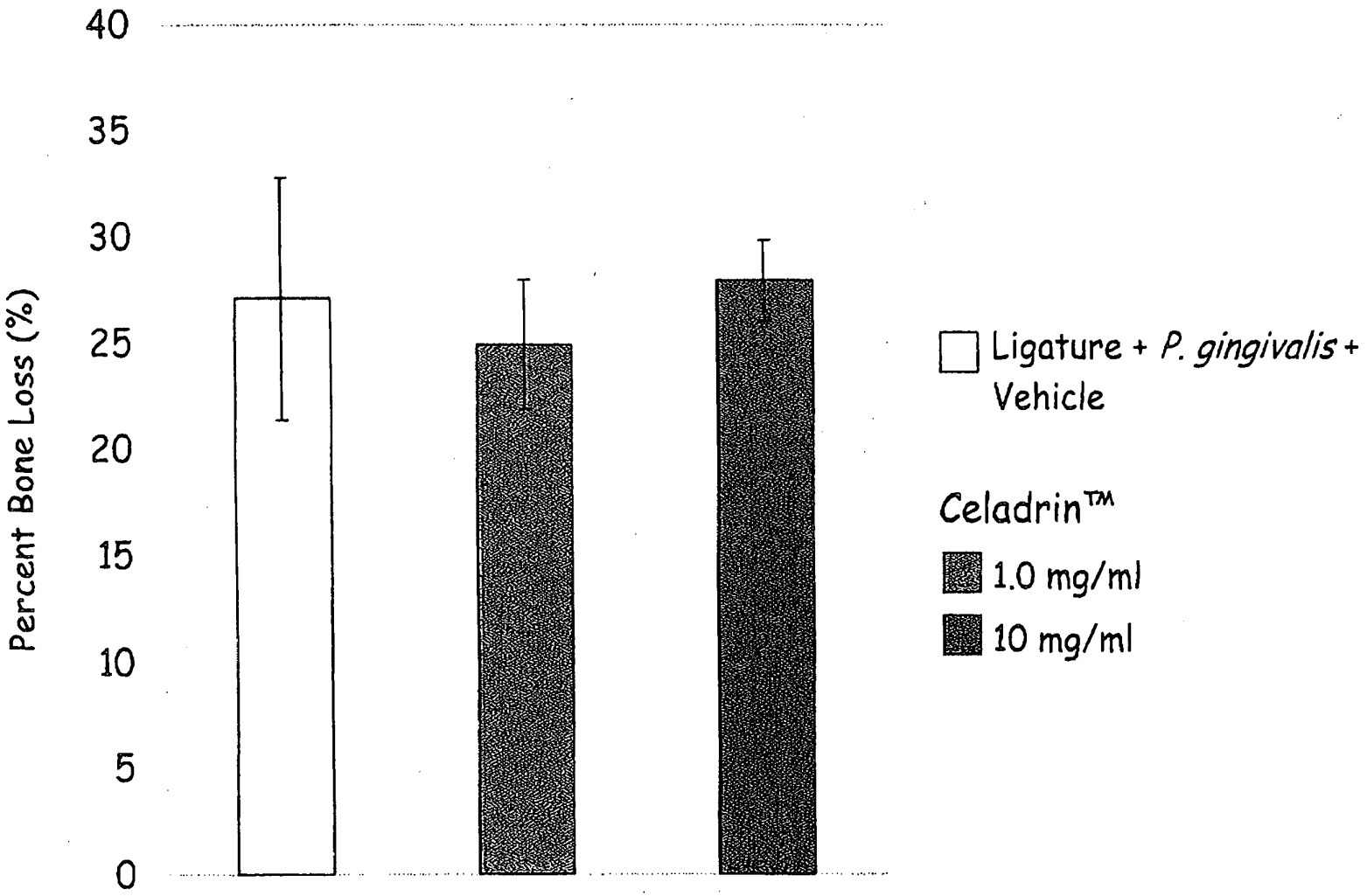
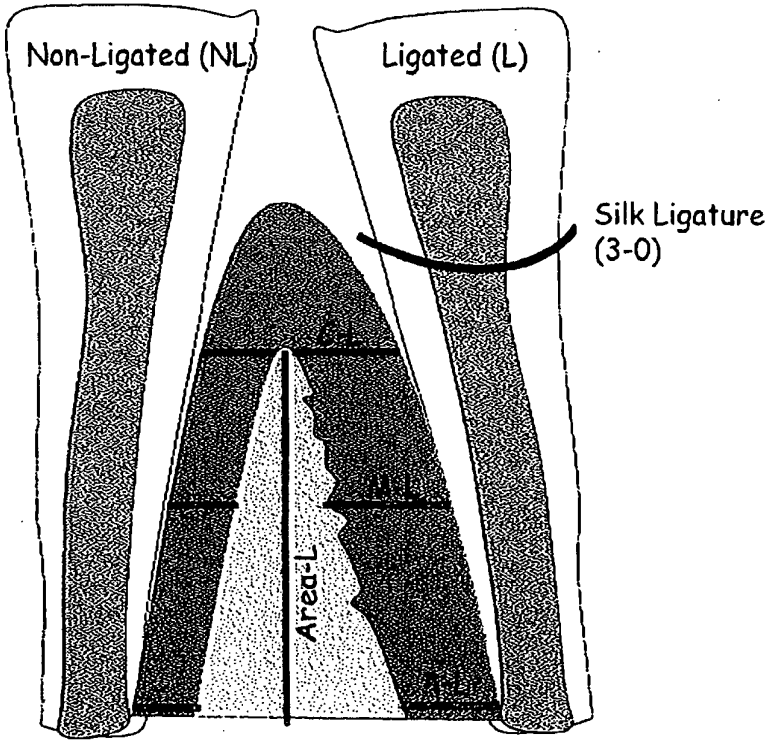


Figure 6

Histomorphometrical Measurements



C-L: Distance between alveolar bone crest and the ligated tooth

M-L: Distance between alveolar bone and the ligated tooth at middle

A-L: Distance between alveolar bone crest and the ligated tooth at apex

Area-L: Area of alveolar bone adjacent to ligated tooth

Figure 7

Histological Bone Loss

(Distance Between Root Surface and Alveolar Bone on the Ligated Side)

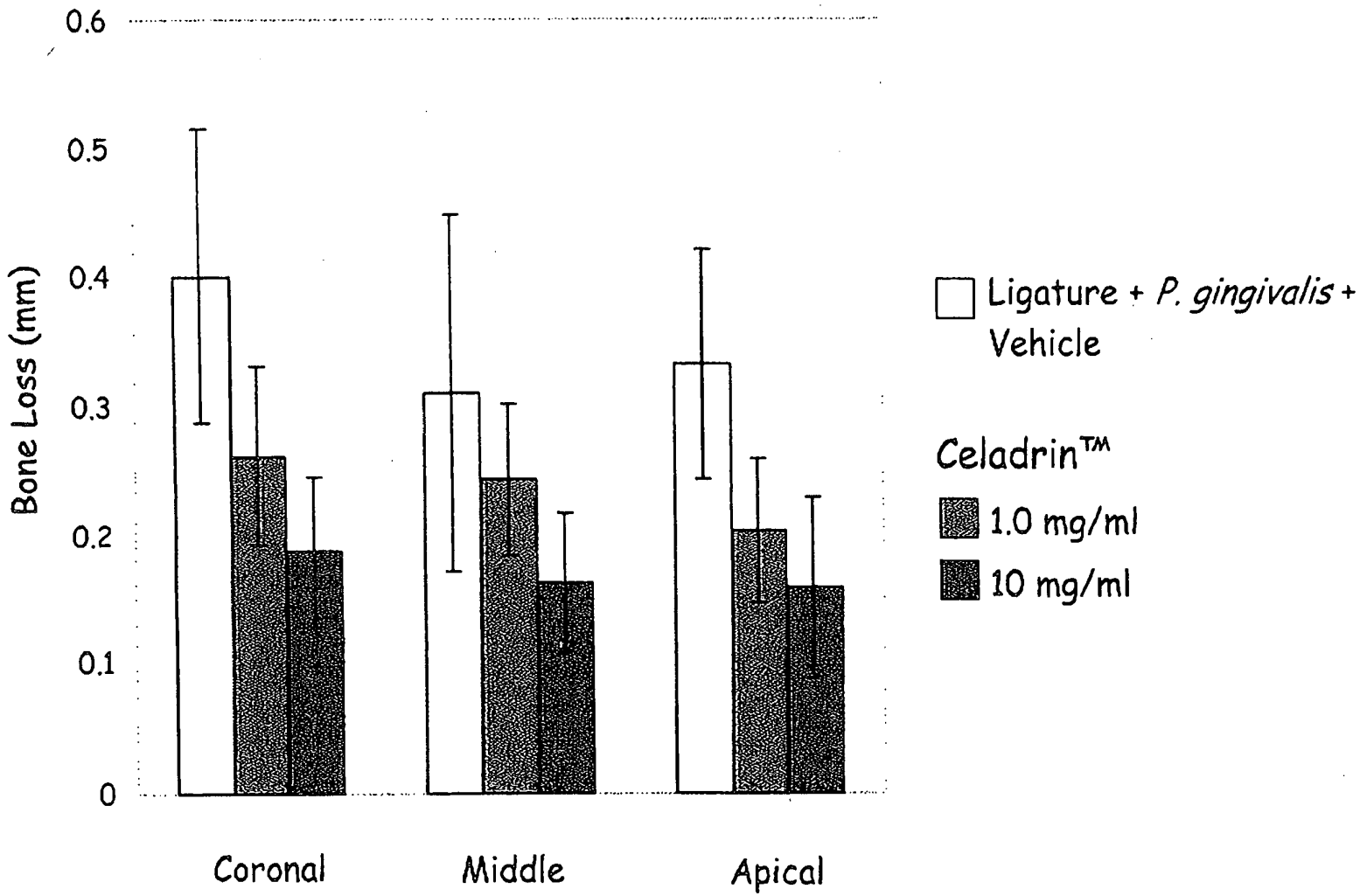


Figure 8

Histological Bone Loss

(Area of Alveolar Bone on the Ligated Side)

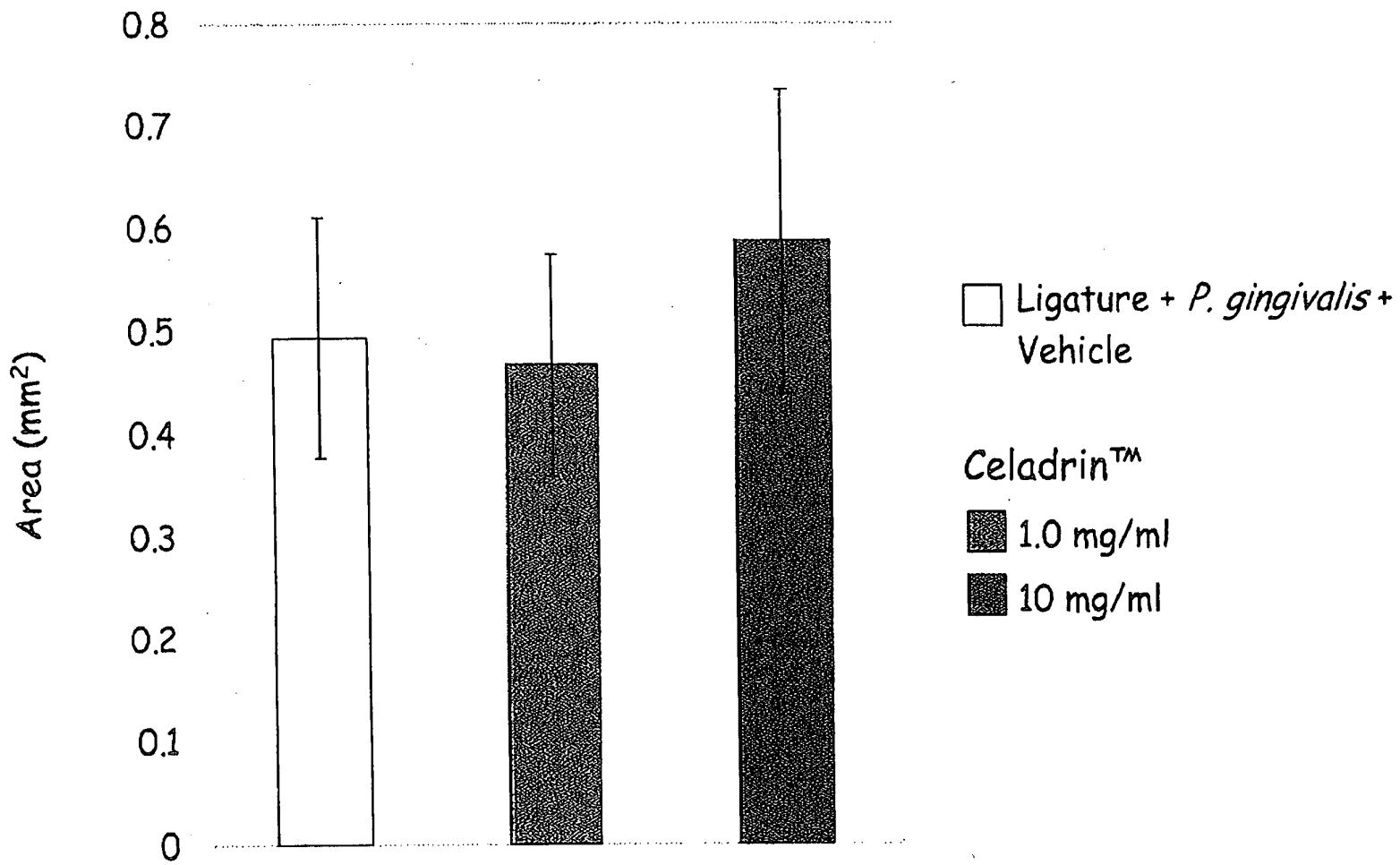
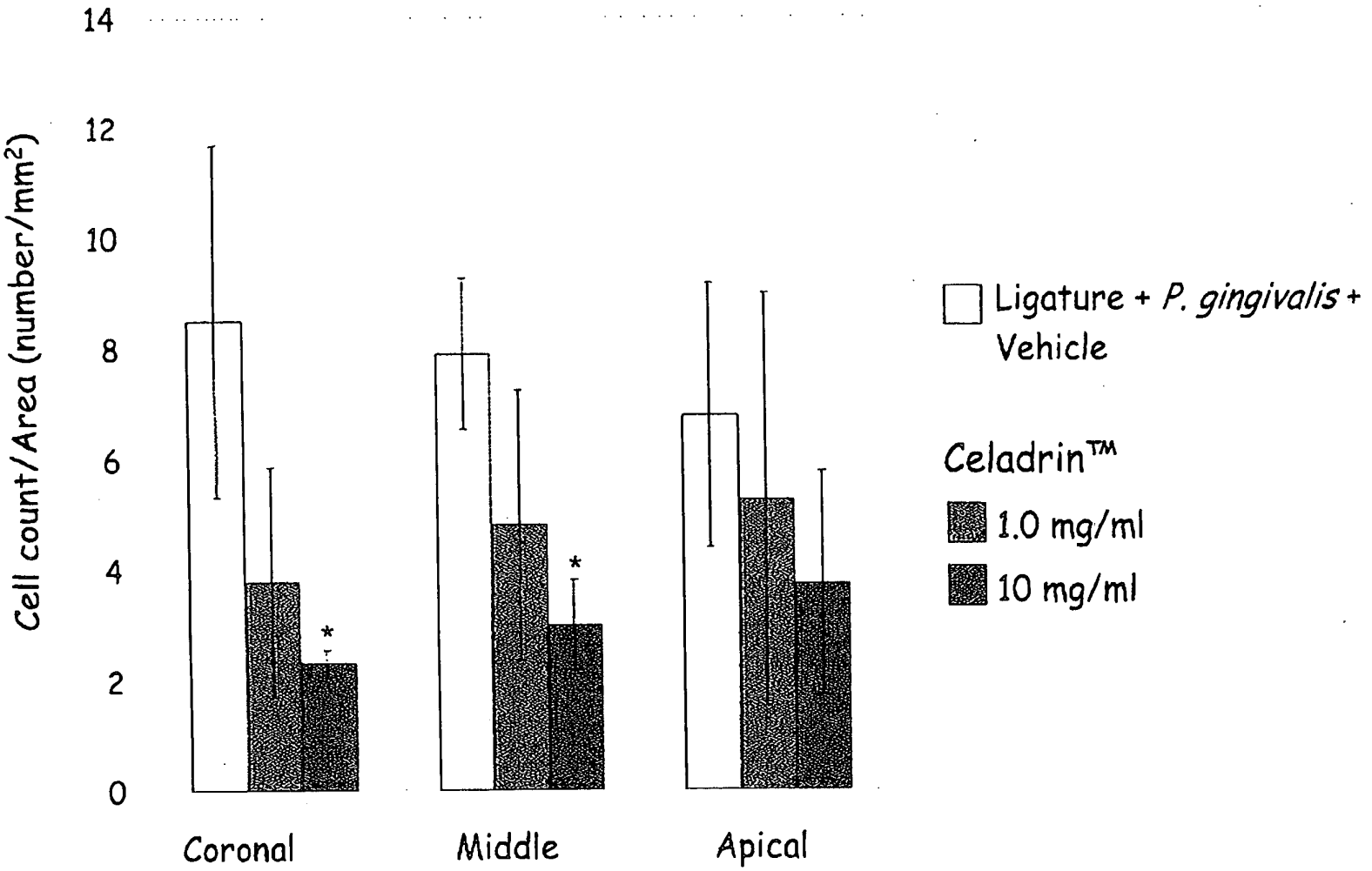


Figure 9

Bone Loss

(Osteoclastic Activity)



*p<0.05 compared to vehicle